

REMARKS**Status of the Claims**

Claims 1 and 61-75 were pending in the application as of the Office Action mailed March 9, 2005. New claims 76-84 are added herein. No new matter is added by way of the present Amendments.

Support for the amendments to claims 1 and 61 in connection with the term "neoplasm" can be found in the specification, for example, at page 5, lines 1-3. The present amendment is made to broaden the claimed invention to relate to neoplasms which include malignant cells as previously claimed.

Support for the amendments to claim 1 in connection with the term "administering" can be found in the specification, for example, at page 7, lines 16-27 and page 37, lines 25-28. The present amendment is made to broaden the claimed invention to relate to administering the claimed polypeptide by any route including the previously claimed route of immunization.

Support for new claims 76-84 can be found in the originally filed claims and in the specification, for example, at page 20, line 24.

Claims 1 and 61-84 are currently pending in the instant application. Reconsideration of the present application is respectfully requested in view of the amendments above and the remarks below.

Rejections Under 35 U.S.C. § 112, First Paragraph, EnablementClaim 1

Claim 1 is rejected under 35 U.S.C. § 112, first paragraph, because the claimed method allegedly is not enabled for reasons already of record with respect to utility in the Office Action mailed July 12, 2004. Applicant respectfully traverses the present rejection for the reasons stated on the record and for the reasons discussed below.

The test for enablement is whether one reasonably skilled in the art can make and use the claimed invention from the disclosures in the patent application coupled with information known in the art without undue experimentation (see, e.g., *In re Wands*, 858 F.2d at 737, 8 USPQ3d at 1404 (Fed. Cir. 1988)). For the reasons on record, Applicant submits that the Examiner has failed to set forth a *prima facie* case that one skilled in the art would doubt the asserted utility of the claimed invention. In addition, Applicant respectfully submits the following arguments.

Apparently, the Examiner questions in the Office Action mailed July 12, 2004, the relative effectiveness of the claimed administering step in "activating cytotoxic T lymphocytes in an animal having a neoplasm that expresses a Her-2/Neu protein" (emphasis added). For example, the Examiner states in the sentence bridging pages 3 and 4 of the Office Action mailed July 12, 2004, "Thus unless tested, it is unpredictable that mice having tumors that express Her/Neu would produce CTLs specific for SEQ ID NO:12 with high affinity" (emphasis added).

Applicant respectfully points out that the affinity of the SEQ ID NO:12 activated CTLs is irrelevant because the affinity is not recited in the claimed invention. Applicant further notes that the test for enablement does not require a demonstration of the relative effectiveness (i.e., high or low affinity) of the claimed method (see, e.g., *In re Brana* 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995)). In particular, Applicant respectfully submits that any level of demonstrated effectiveness is sufficient to enable the claimed invention (emphasis added).

In this regard Applicant respectfully submits Lustgarten et al. (2004) European Journal of Immunology 34:752-761 (attached herewith for the convenience of the Examiner). Lustgarten et al. demonstrates that immunization of Neu transgenic mice, which overexpress Her-2/Neu and develop spontaneous mammary tumors, with SEQ ID NO:12 (referred to as p773-782 in the reference) results in the activation of CTLs that specifically recognize Her-2/Neu with at least low avidity (see, e.g., page 753, column 1, line 25, which states, "they contain a T cell repertoire of low avidity"). Accordingly, the instant reference demonstrates the claimed invention, namely that administering the polypeptide of SEQ ID NO:12 to an animal having a neoplasm that expresses a Her-2/Neu protein specifically activates cytotoxic T lymphocytes in the animal.

Applicant respectfully requests that the present rejection of claim 1 be withdrawn in view of the arguments on record and in view of the newly cited evidence from Lustgarten et al. that administration of the polypeptide of SEQ ID NO:12 results in

the specific activation of cytotoxic T lymphocytes in an animal having a neoplasm that expresses a Her-2/Neu protein.

Claims 61-75

Claims 61-75 are rejected under 35 U.S.C. § 112, first paragraph, because the claimed method of independent claim 61 allegedly is not enabled for reasons already of record in the Office Action mailed July 12, 2004, which reasons are reiterated in the Office Action mailed February 9, 2005 (hereinafter the present Action). Applicant respectfully traverses the present rejection for the reasons stated on the record and for the reasons discussed below.

On page 3 of the present Action, the Examiner alleges that "one of skill in the art would be forced into undue experimentation to practice the claimed invention" because treating a neoplasm having cells that express a Her-2/Neu protein in a patient by a method comprising administering a polypeptide having the amino acid sequence of SEQ ID NO:12 is alleged unpredictable based upon the amount of disclosure in the specification and the knowledge in the art. For the reasons on record, Applicant does not concede that the Examiner has established a reasonable basis for why one skilled in the art would question the asserted enablement of the claimed invention and, thus, the Examiner has not established a *prima facie* case of lack of enablement.

Furthermore, in the Office Action mailed March 9, 2005, the Examiner has set the standard for the relative effectiveness of the claimed administering step arbitrarily

high because throughout the present Office Action the Examiner states that there allegedly is no evidence of tumor "killing" (emphasis added).

Applicant does not concede that the efficacy of the claimed invention in treating a tumor in a patient having an Her-2/Neu tumor must have been demonstrated *in vivo* on the priority date of the present application for reasons of record and because the enablement requirement does not require a working example and because treatment of Her-2/Neu tumor cells *in vitro* was demonstrated in the specification as discussed on the record. There is no requirement for meeting the enablement requirement that the claimed results be tested in a patient having a Her-2/Neu tumor. As stated by the Federal Circuit:

"If applicants were required to wait until an animal naturally developed this specific tumor before testing the effectiveness of a compound against the tumor *in vivo*, as would be implied from the Commissioner's argument, there would be no effective way to test compounds *in vivo* on a large scale". See *In re Brana* at 1440.

For the reasons discussed above, there can be no requirement that there be a demonstration of killing the tumor in a patient wherein the tumor expresses a Her-2/Neu protein in part because the specification demonstrated efficacy in an appropriate tumor model system which was an *in vitro* model of *in vivo* treatment of a tumor (as discussed on the record).

Applicant also traverses the Examiner's apparent requirement that the standard of efficacy is tumor killing. Applicant respectfully submits that any level of efficacy would meet the standard for enablement of the pending claims. For example, Lustgarten et al. (see above) demonstrate inhibition of tumor growth which would be one manner of measuring the efficacy of the claimed invention.

Still further, Lustgarten et al. demonstrate that CTLs activated by SEQ ID NO:12 reject implanted N202.A2 cells (Her-2/Neu expressing tumor cells) by adoptive transfer (see, e.g., page 754 columns 1 and 2). In other words, CTLs activated by SEQ ID NO:12 from a first mouse are able to reject Her-2/Neu expressing neoplasms in a second mouse when the activated CTLs are transferred to that second mouse. The data show that a single transfer of SEQ ID NO:12 activated CTLs inhibited 10-15% of tumor growth, two transfers inhibited 25-30% of tumor growth, and three transfers inhibited 40-45% of tumor growth (see, e.g., page 755, column 1). Thus, Lustgarten et al. demonstrates that CTLs activated by administration of SEQ ID NO:12 inhibit Her-2/Neu expressing neoplasms *in vivo*.

Accordingly, Lustgarten et al. demonstrates that the claimed invention is enabled as described in the specification, namely that administering the polypeptide of SEQ ID NO:12 to a patient having a neoplasm that expresses a Her-2/Neu protein provides a method of treating the patient having the neoplasm.

Applicant respectfully requests that the present rejection of claims 61-75 be withdrawn in view of the arguments on record, the arguments above, and in view of the newly cited

evidence from Lustgarten et al. that administration of the polypeptide of SEQ ID NO:12 results in the specific activation of cytotoxic T lymphocytes (CTLs) in a patient having a neoplasm that expresses a Her-2/Neu protein and that the specifically activated CTLs treat a neoplasm in a patient in need of treatment for said neoplasm.

In a miscellaneous matter, the Examiner refers to SEQ ID NO:10 at page 7, line 2 and page 12, line 2 of the present Action. Applicant assumes that the Examiner meant to refer to SEQ ID NO:12.

Next the Examiner asserts that claims 61-75 allegedly encompass a tumor which term "tumor" allegedly means any swelling, growth, or enlargement of tissue such as splenitis, adipose, etc... wherein cells of said tissue express a Her-2/Neu protein (citing Stedman's Medical Dictionary 25th ed (1990) pages 1652-1653 for the definition of tumor). The Examiner further asserts that the specification allegedly does not teach how to treat such tumor.

Applicant does not concede to the Examiner's suggested dictionary definition of the term "tumor" because the specification states that the invention is related to cancer, tumors, neoplasia, etc. which make it clear that the claims are related to cancerous tumors (see, e.g., page 5, lines 1-3). However, in view of the amendment of claims 1 and 61 to relate to a neoplasm, the present rejection is believed to be moot and Applicant respectfully requests that it be withdrawn.

CONCLUSION

Claims 1 and 61-84 are pending in the present application. Applicant believes that claims 1 and 61-84 are in condition for allowance and earnestly solicits an early notification of allowance from the Examiner.

If there are any additional fees (or overpayments) associated with this Response, or any Response associated with this application, the Director is hereby authorized to charge (or credit) our Deposit Account No. 19-0962.

Respectfully submitted,

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Date

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The CD8⁺ T cell repertoire against Her-2/neu antigens in neu transgenic mice is of low avidity with antitumor activity

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The majority of tumor-associated antigens are aberrantly expressed or overexpressed normal gene products. Therefore, mechanisms responsible for self tolerance dampen immune responses against these antigens. To evaluate the effect that tolerance has on the immune responses against tumor antigens, we characterized the CD8⁺ T cell responses in neu mice. T cell responses against the A2.1/neu p369–377 and p773–782 peptides were evaluated in neu mice that were crossed with A2.1/Kb transgenic mice (A2xneu). Tetramer binding and cytotoxic activity demonstrate that, compared to CTL from A2.1/KbxFVB wild-type mice (A2xFVB), CD8⁺ T cells from A2xneu mice were of lower avidity for the peptides. Despite the fact that A2xneu mice are tolerant, multiple immunizations with DC pulsed with the p369–377 or p773–782 peptides in the presence of IL-2 retarded tumor growth in A2xneu mice, and immunizations in combination with the anti-OX40 mAb further enhanced the antitumor response. Taken together, these data indicate that low-avidity T cells for neu antigens persisting in A2xneu mice have the capacity to develop antitumor responses as long as they are provided with efficient costimulation. These results underscore the potential role of low-avidity T cells in antitumor immunity and may offer an important component for vaccination immunotherapies.

Key words: Tumor immunology / Tolerance / Vaccination / CTL / Antigens/peptides

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1 Introduction

The discovery of tumor-associated antigens (TAA) [1, 2] has been an important breakthrough in tumor immunology, because it is possible to devise immunotherapeutic approaches to promote T cell responses against such antigens and induce a protective immunity against neoplastic malignancies [3]. The majority of the currently defined TAA are often overexpressed products of normal cellular genes [4]. As such, these overexpressed proteins pose a significant challenge to the design of effective T cell immunotherapies as self tolerance has to be considered [5]. Based on transgenic mouse models, it is now clear that tolerance is capable of deleting reactive high-avidity T cells against the transgene (self), thereby leading to self tolerance [6]. However, T cell elimination through tolerance is not absolute, since self-specific T cells can be isolated from tolerant hosts [7, 8]. Moreover, it has been demonstrated that low-avidity T cells can be activated, expanded and involved in antitumor

responses [9, 10]. Thus, the observation that low-avidity T cells persist *in vivo* and that they can recognize self antigens underscores their potential role in antitumor immunity.

The significance of understanding the mechanism responsible for the persistence of low-avidity T cells relates not only to our understanding of autoimmunity, but also to the use of such cells to target self tumor antigens for tumor destruction. Therefore, a central question is whether the available repertoire of T cells specific for up-regulated self tumor antigens is sufficient in number or avidity to mount an effective antitumor response. We have addressed this fundamental question in an experimental model in which the rat *neu* protooncogene is expressed in the mammary tissue under the control of the MMTV promoter (FVB-MMTV-*neu* transgenic mice) [11, 12]. Neu mice develop spontaneous mammary tumors, and the multi-step process for the development of mammary tumors in these animals recapitulates the clinical progression and pathogenesis of the human disease. Thus, this model is ideal for the evaluation of immunological responses against *neu* antigens as well as the evaluation of novel immunotherapeutic modalities for tumor eradication.

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Abbreviation: TAA: Tumor-associated antigen

Advances in understanding how T cells become activated have led to the development of new strategies to enhance antitumor responses. One commonly used therapy is the application of IL-2, which can mediate a wide range of immune effects such as stimulating and activating T and NK cells and increasing T cell infiltration into tumors [13, 14]. Accumulative evidence indicates that the use of other costimulatory molecules, such as the tumor necrosis factor receptor (TNFR) family, can also enhance and augment the immunogenicity of the tumor. It has been demonstrated that engagement with the OX40 ligand (OX40L) or anti-OX40 mAb delivers signals to OX40⁺ T cells, prolonging and propagating T cell responses [15]. Moreover, antibodies against OX40 rescue effector T cells from activation-induced cell death [16] and enhance antitumor immune responses *in vivo* [17, 18].

We have previously identified two HLA-A2.1/neu-restricted immunodominant epitopes (p369–377 and p773–782) [19]. To evaluate peptides specific for these epitopes, neu mice were crossed with A2.1/Kb transgenic mice [20] (A2×neu), and tetramer binding and cytotoxic activity were evaluated. The results indicate that A2×neu mice are functionally tolerant to neu; however, they contain a T cell repertoire of low avidity when compared to the repertoire of A2×FVB mice (FVB mice crossed with the A2.1/Kb mice). On the basis that a low-avidity repertoire persists in A2×neu mice, we examined the antitumor potential of these cells. Our results show that only multiple immunizations with pulsed DC in the presence of IL-2 induce an antitumor response which delays tumor growth in A2×neu mice. Furthermore, A2×neu mice immunized with DC in the presence of anti-OX40 mAb induce an antitumor response resulting in substantial tumor reduction. This enhancement was due to a greater number of expanded p369–377- and p773–782-specific CD8⁺ T cells. Taken together, these results indicate that the low-avidity T cell repertoire for a self tumor antigen is functional, can be activated and expanded with the help of costimulation, and that these cells have the potential to provide a significant therapeutic benefit.

2 Results

2.1 Comparison of A2-neu-specific CTL responses between A2×neu and A2×FVB mice

Although previous reports have suggested that neu mice are tolerant to neu antigens [21–23], there are no available data on the evaluation of specific T cell responses. neu and FVB mice were crossed with A2.1/Kb transgenic mice, and T cell responses against the A2.1/neu

p369–377 or p773–782 peptides were evaluated. Both the A2×neu and A2×FVB mice were immunized with DC pulsed with these peptides. Spleen cells were stimulated *in vitro* and analyzed for their ability to bind the A2.1-p369–377-PE and A2.1-p773–782-PE tetramers. As shown in Fig. 1A and B, CD8⁺ T cells from A2×FVB mice bind the tetramers with higher intensity compared to CD8⁺ T cells from A2×neu mice. The tetramers do not stain the HIV-POL CTL and no A2.1-p773–782-PE tetramer binding is detected in animals immunized with the p369–377 peptide or vice versa, demonstrating that binding to the tetramer is specific.

Stimulated spleen cells were analyzed for their lytic activity in a peptide dose curve assay. Compared to CTL from A2×FVB mice, CTL obtained from A2×neu mice demonstrated significantly lower recognition of both the p369–377 (Fig. 1C) and p773–782 (Fig. 1D) peptides. As a control for specificity, we used a restricted HLA-A2.1/HIV-POL CTL line [19], demonstrating that recognition of the neu-restricted CTL is specific. Taken together, these results strongly indicate that T cells from A2×neu mice are hyporesponsive to neu antigens.

2.2 CTL from A2×neu mice are of low avidity

The preceding results demonstrated that after immunization, the cytotoxic activity of the CD8⁺ T cells from A2×FVB mice was superior to the cytotoxic activity of CD8⁺ T cells from A2×neu mice. This raises the question whether the lack of an optimal response in A2×neu mice to immunodominant peptides was due to a quantity response (not enough p369–377 and p773–782 T cells existing in the repertoire) or a quality response (p369–377 and p773–782 CTL from A2×neu mice do not effectively recognize immunodominant peptides). Tetramer binding and cytotoxic activity were analyzed for p773–782 CTL lines derived from A2×neu and A2×FVB mice. The p773–782 CTL were stained with serial dilutions of the A2.1-p773–782-PE tetramer (1:50, 1:250 and 1:2,500). As shown in Fig. 2A, p773–782 CTL from A2×FVB mice demonstrate a stronger binding activity, while a weaker binding activity is detected in p773–782 CTL from A2×neu mice. We also analyzed TCR and CD8 expression of these CTL (Fig. 2B), and FACS analysis confirmed that CTL from both A2×neu and A2×FVB mice have the same level of expression of these molecules. These results suggest that the lower response of CTL from A2×neu mice could not be attributed to low expression of TCR and CD8 molecules. The CTL lines from A2×FVB mice showed a higher cytotoxic activity than CTL from A2×neu mice (Fig. 2C). Taken together, tetramer binding and cytotoxic activity show that there is a correlation, demonstrating a difference in the T cell avid-

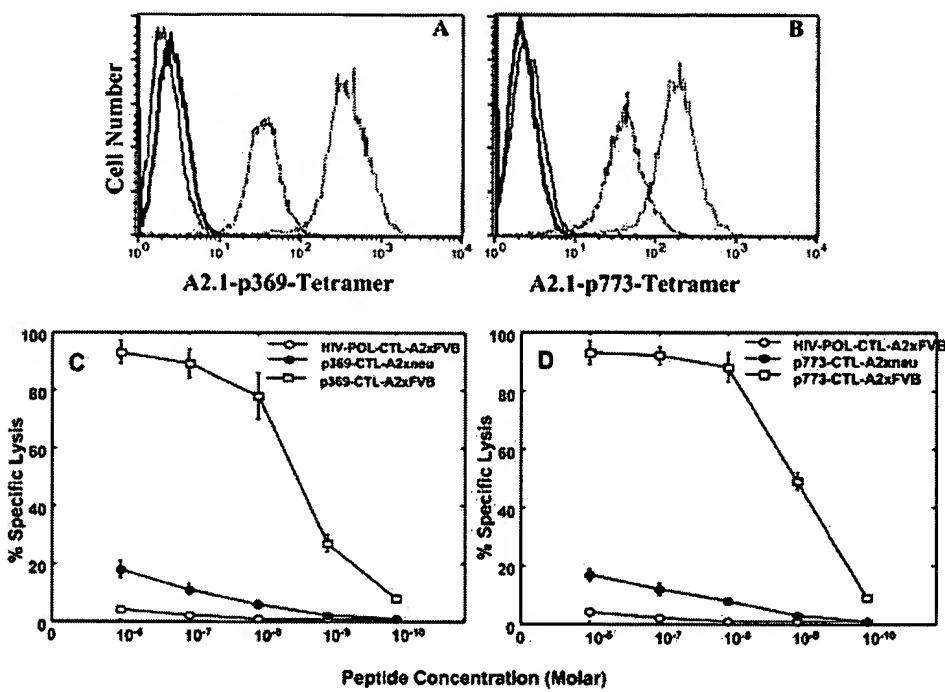


Fig. 1. The T cell repertoire of A2xneu mice is hyporesponsive to immunodominant epitopes. A2xneu and A2xFVB mice were immunized with DC pulsed with the p369-377 and p773-783 peptides, and restimulated T cells were stained with the A2.1-p369-377-PE and A2.1-p773-782-PE tetramers and anti-CD8-FITC. Viable CD8⁺ cells were gated, and the fraction of cells stained with tetramers was analyzed. (A) Staining with A2.1-p369-377-PE tetramer. Thin line; p773-782 CTL. Thick line; HIV-POL CTL. Broken line; p369-377 CTL from A2xneu mice. Dotted line; p369-377 CTL from A2xFVB mice. (B) Staining with A2.1-p773-782-PE tetramer. Thin line; p369-377 CTL. Thick line; HIV-POL CTL. Broken line; p773-782 CTL from A2xneu mice. Dotted line; p773-782 CTL from A2xFVB mice. Lytic activity of spleen cells from p369-377 (C) and p773-782 (D) peptide-immunized animals. Stimulated spleen cells were assayed at an E:T ratio of 10:1 for cytotoxicity against T2-A2/Kb target cells pulsed with their respective peptides using decreasing concentrations of the peptides. The A2.1-HIV-POL-restricted CTL were used as a control. Data are the means of four individually analyzed mice per group \pm SD. The results shown are representative of three independent experiments.

ity between A2xneu and A2xFVB mice for the recognition of neu antigens, and indicating that A2xneu mice contain only low-avidity T cells for neu antigens.

2.3 CTL from A2xneu mice recognize neu antigens on tumors cells

We tested the ability of the p369-377- and p773-782-restricted CTL from both A2xneu and A2xFVB mice to recognize and kill tumors expressing HLA-A2.1 and neu molecules. For these experiments, we established a cell line (N202.A2) generated from a spontaneous tumor in A2xneu mice. The N202.A2 cells expressed A2.1 and neu molecules (Fig. 3A). As a control, we used the N202 cells, a cell line generated from a spontaneous tumor from neu mice [24] that does not express A2.1 molecules (Fig. 3A). CTL from A2xneu mice were capable of recognizing N202.A2 targets, albeit at significantly lower levels

than CTL from A2xFVB mice (Fig. 3B). The CTL did not recognize N202 cells, indicating that they recognized A2-neu-restricted antigens expressed on tumor cells.

2.4 Adoptive transfer of low-avidity T cells delays tumor growth

Having demonstrated that CTL from A2xneu recognize the N202.A2 cells, we wanted to evaluate whether these CTL would have an antitumor effect. We confirmed that N202.A2 cells formed tumors in A2xneu mice but not in A2xFVB mice (data not shown). We analyzed the ability of A2xneu-derived CD8⁺ T cells to reject N202.A2 cells after adoptive transfer. A critical question was how to maximize the antitumor activity of the low-avidity T cells. To this end, we tested whether multiple transfers would enhance the antitumor immune response. We compared animals that had received one, two or three transfers. A

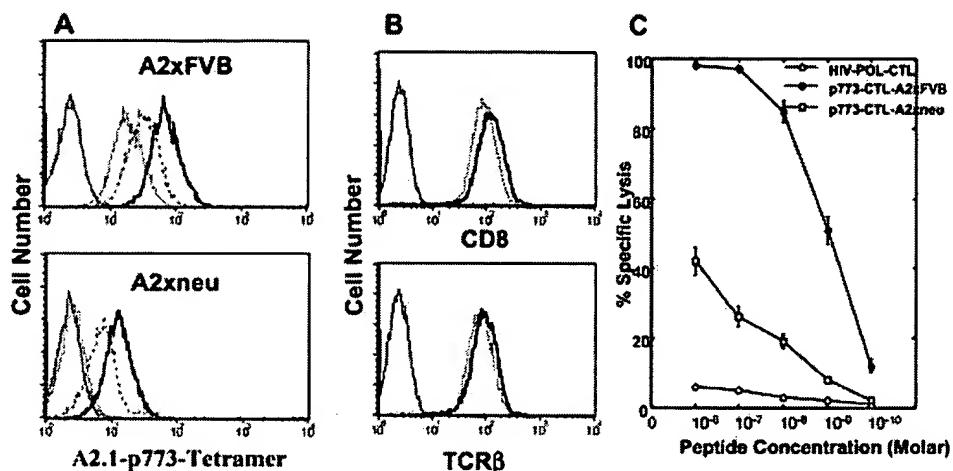


Fig. 2. CTL from A2xneu mice have low-avidity TCR. p773-782 CTL lines were established from A2xFVB and A2xneu mice. (A) CTL were stained with serial dilutions of A2.1-p773-783-PE tetramer: 1:50 thick line (2 µg); 1:250 dotted line; 1:2500 broken line; and the solid thin line shows cells stained with A2.1-p369-377-PE tetramer (2 µg) as a control. (B) CTL were analyzed for the surface expression of CD8 and TCR molecules. The thick line corresponds to CTL derived from A2xFVB mice, the broken line corresponds to CTL derived from A2xneu mice, and the thin line corresponds to unstained control. (C) Cytotoxic activity of CTL was analyzed against T2-A2.1/Kb target cells pulsed with decreasing concentrations of p773-782 peptide at an E:T ratio of 10:1. A2.1-HIV-POL-restricted CTL were used as a control. Similar results were found with p369-377 CTL (data not shown). Data presented are the means of two independent experiments \pm SD.

single transfer of CTL derived from A2xFVB mice rejected the tumor (Fig. 4A). Interestingly, three transfers of the p369-377 (Fig. 4B) or p773-782 CTL (Fig. 4C) inhibited 40–45% of tumor growth, while fewer transfers demonstrated a lower efficiency for tumor growth inhibition (two transfers inhibited 25–30% and a single transfer inhibited 10–15% of tumor growth). These results demonstrate that the low-avidity CTL could delay tumor growth, indicating that these cells contribute to the antitumor response.

2.5 Immunization of A2xneu mice induces an antitumor response

The preceding results demonstrate that CTL derived from A2xneu mice have the capacity to recognize and kill the N202.A2 tumor cells *in vitro* and *in vivo*. Next, we evaluated whether immunization of A2xneu mice would induce an immune response capable of delaying growth or rejection of an established tumor. We compared animals that were immunized once, twice or three times with DC pulsed with p369-377 or p773-782 peptides in the presence of IL-2. Animals immunized three times with DC pulsed with p369-377 (Fig. 5A) or p773-782 (Fig. 5B) peptide showed ~25–30% tumor growth inhibition, while two immunizations induced ~12–15% tumor growth inhibition and one injection resulted in ~7–9% tumor growth inhibition (data not shown). We confirmed

that the antitumor responses were CD8⁺ T cell dependent since treatment with anti-CD8 mAb abrogated the antitumor response. These results suggest that, although the repertoire of neu mice is of low affinity, this repertoire can be activated and has antitumor potential.

2.6 Anti-OX40 mAb enhances the antitumor response in A2xneu mice

In the last few years, OX40 has gained importance as a costimulatory molecule capable of expanding immune responses and enhancing the antitumor immune responses of animals with established tumors [17, 18]. We evaluated whether the combination of DC-based vaccine and anti-OX40 mAb would stimulate a stronger antitumor response. As shown in Fig. 6, DC-based vaccines in the presence of anti-OX40 mAb induced a significantly stronger protective antitumor response resulting in ~45–50% tumor growth inhibition, while DC-based immunization in the absence of anti-OX40 mAb only inhibited ~25% of tumor growth. A weaker response was observed in animals that received either one or two immunizations with pulsed DC in the presence of IL-2 and anti-OX40 mAb (data not shown). Mice immunized three times with DC pulsed with HLA-A2.1/HIV-POL peptide in the presence of IL-2/anti-OX40 mAb showed minimal protection (~7% tumor growth inhibition), indicating the specificity of the antitumor responses induced by the

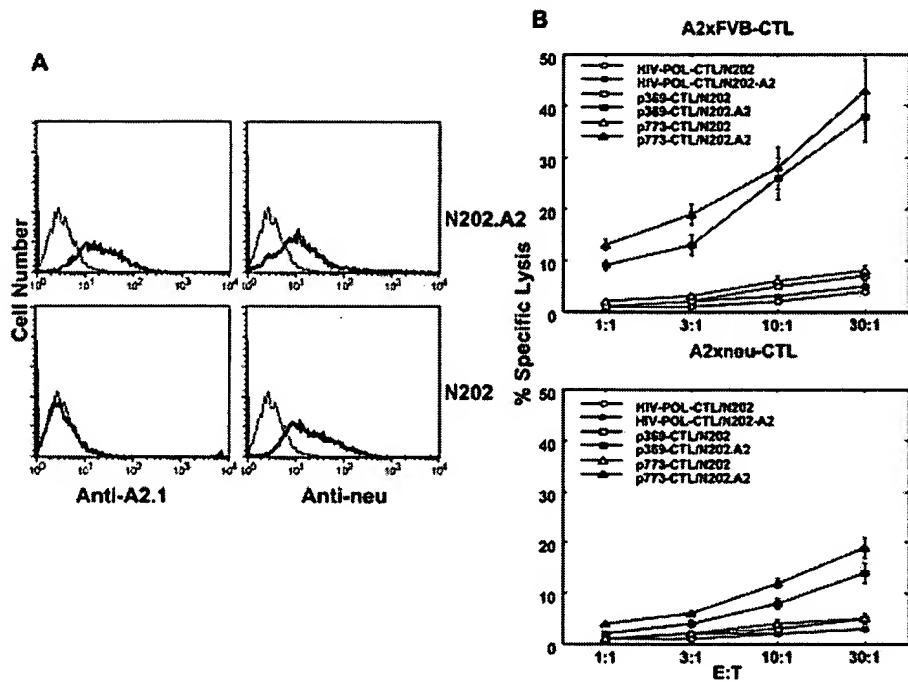


Fig. 3. Lysis of N202.A2 cells by p369-377 and p773-782 CTL. (A) N202.A2 and N202 cells were analyzed for the expression of HLA-A2.1 and neu. (B) The p369-377 and p773-782 CTL from A2xFVB and A2xneu mice were assayed for the specific killing activity of ^{51}Cr -labeled N202.A2 and N202 cells. Data presented are the means of three independent experiments \pm SD.

p369-377 (Fig. 6A) or p773-782 (Fig. 6B) peptides. These results demonstrate that it is possible to manipulate the immune response in A2xneu mice and to considerably enhance the antitumor responses in tolerant hosts.

To evaluate the effect of anti-OX40 mAb on the antitumor response, we tested both tetramer binding and cytotoxic activity. As shown in Table 1, A2xneu mice immunized with DC pulsed with peptides in the presence of anti-OX40 mAb showed a higher number of spleen cells that bind the A2.1-p369-377-PE or A2.1-p773-782-PE tetra-

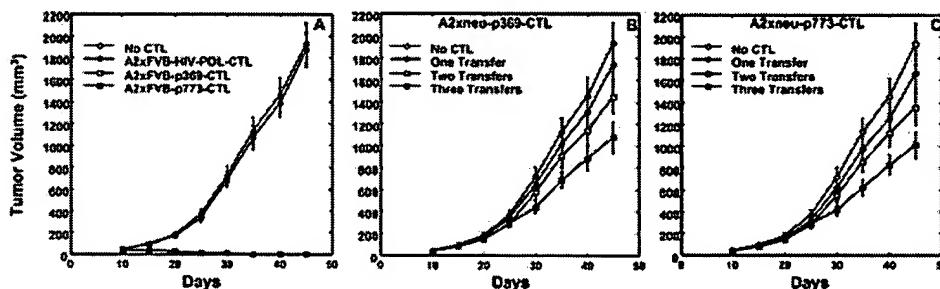


Fig. 4. Adoptive transfer of neu-reactive CTL from A2xneu mice delays growth of established tumors. N202.A2 cells (10^6) were injected s.c. in A2xneu mice on day 0. Animals received one (day 7), two (days 7 and 17) and three (days 7, 17 and 27) intratumoral transfers of *in vitro*-activated CTL. Each transfer consisted of 10^7 CTL. Animals were supplemented with daily i.p. injections of IL-2 (10^4 IU/day) for 1 week after each transfer. (A) Animals were transferred once with p369-377 or p773-782 CTL derived from A2xFVB mice. As a control for specificity, animals were transferred three times with the A2.1-HIV-POL-restricted CTL derived from A2xFVB mice. (B) Animals were transferred one, two or three times with p369-377 CTL derived from A2xneu mice. (C) Animals were transferred one, two or three times with p773-782 CTL derived from A2xneu mice. Data are the means of two independent experiments \pm SEM with at least five animals per group per experiment. A significant ($p < 0.05$, Student's *t*-test) difference was found between mice that had received no CTL and mice that had received three transfers.

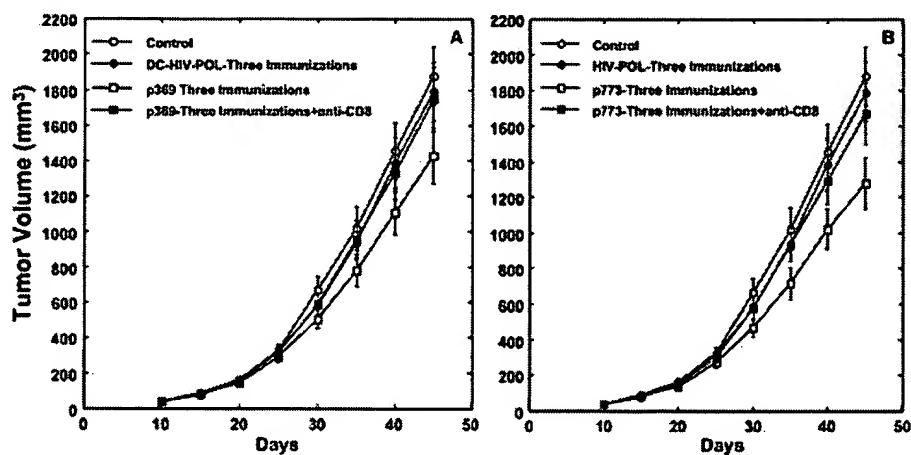


Fig. 5. Multiple immunizations with DC induce an antitumor response in A2xneu mice. A2xneu mice were inoculated s.c. on day 0 with 10^6 N202.A2 cells, and 1 week later animals were immunized as follows: Animals received one (on day 7), two (on days 7 and 17) and three (on days 7, 17 and 27) immunizations. Each immunization consisted of an s.c. injection of 10^6 DC pulsed with the peptides. Animals were supplemented with daily i.p. injections of IL-2 (10^4 IU/day) for 1 week after each immunization. (A) Animals were immunized one, two or three times with DC pulsed with the p369-377 peptide in the presence of IL-2. As a control, a group of animals was immunized three times with 10^6 DC pulsed with A2.1-HIV-POL peptide in the presence of IL-2. (B) Animals were immunized one, two or three times with DC pulsed with the p773-782 peptide in the presence of IL-2. Data are the means of two independent experiments \pm SEM with at least five animals per group per experiment. A significant ($p < 0.25$, Student's *t*-test) difference was found between the control group and mice that had received three immunizations.

mer as compared to animals without anti-OX40 mAb. We observed that immunization in the presence of IL-2, in contrast to anti-OX40 mAb, did not significantly augment the number of tetramer-binding cells. The p369-377 and

p773-82 peptide-specific cytotoxic T cell responses were also augmented in animals treated with anti-OX40 mAb but minimally increased with IL-2 (Table 1).

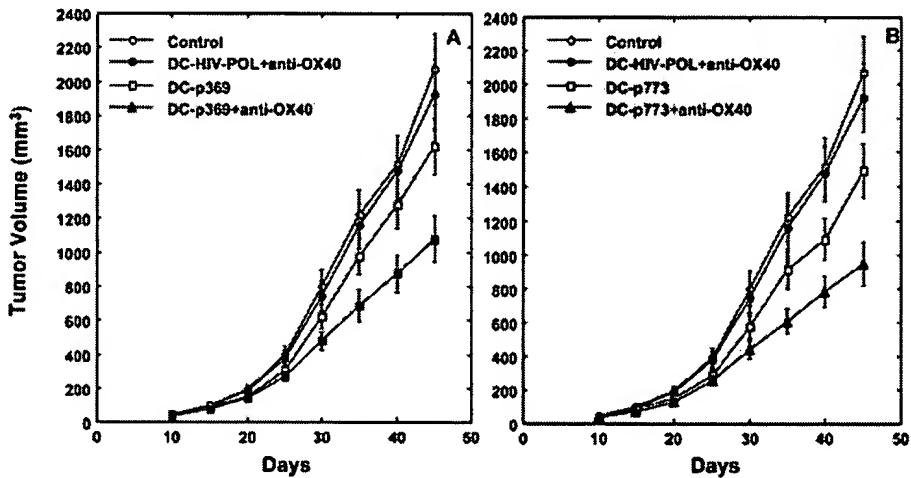


Fig. 6. Anti-OX40 enhances the antitumor response in A2xneu mice. A2xneu mice were inoculated s.c. on day 0 with 10^6 N202.A2 cells. Animals were immunized three times (on days 7, 17 and 27) with s.c. injections of 10^6 DC pulsed with the peptides. Animals were supplemented with daily i.p. injections of IL-2 (10^4 IU/day) for 1 week after each immunization. Anti-OX40 (100 μ g/injection) was administered 2 days after each immunization. Data are the means of six to eight animals per group \pm SEM. A significant ($p < 0.01$, Student's *t*-test) difference was found between the control group and mice that had received three immunizations in the presence of anti-OX40 mAb.

Table 1. Effect of costimulatory molecules on the immune response of A2 \times neu mice

Immunization ^{a)}	% Tetramer/CD8 ^{b)}		Cytotoxic activity ^{c)}	
	p369–377 CTL	p773–782 CTL	p369–377 CTL	p773–782 CTL
DC-pep	1.2 \pm 0.2	2.1 \pm 0.3	12 \pm 3	15 \pm 2
DC-pep+IL-2	1.7 \pm 0.4	2.9 \pm 0.5	19 \pm 4	23 \pm 3
DC-pep+anti-OX40	4.4 \pm 0.7	6.8 \pm 0.9	34 \pm 4	42 \pm 6
DC-pep+IL-2+anti-OX40	4.8 \pm 0.8	7.1 \pm 1.2	37 \pm 6	45 \pm 5

^{a)} A2 \times neu mice ($n=3$ –5 mice/group) were immunized with DC pulsed with p369–377 or p773–782 peptides in the presence or absence of IL-2 (administered daily 10⁴ IU/day i.p. for 1 week starting 1 day after immunization) and anti-OX40 mAb (single injection of 100 μ g starting 2 days after immunization).

^{b)} Ten days after immunization, animals were killed and spleen cells were stained with the A2.1-p369–377-PE or A2.1-p773–782-PE tetramer and anti-CD8-FITC Ab. Results are expressed as % (\pm SD) of tetramer-binding/CD8 $^{+}$ cells for the mean of individual mice analyzed.

^{c)} Portion of the same spleens used for tetramer staining were restimulated *in vitro* and cells were tested for cytotoxicity against ⁵¹Cr-labeled T2-A2.1/Kb target cells pulsed with p369–377 or p773–782 peptides at an E:T ratio of 10:1. Results are expressed as % of specific lysis \pm SD for the mean of individual mice analyzed.

3 Discussion

The majority of TAA are self antigens and, therefore, T cell tolerance handicaps the immune response against such antigens. Understanding the behavior of T cell responses against self tumor antigens is of great importance for the development of effective immunotherapeutic strategies toward a treatment of cancer. To assess the effect of tolerance against neu responses, T cell responses against the p369–377 and p773–782 A2.1-neu-restricted peptides were evaluated in A2 \times neu mice. The analysis of the T cell responses demonstrates that A2 \times neu CD8 $^{+}$ T cells bind with lower efficiency and intensity to the A2.1-p369–377-PE or A2.1-p773–782-PE tetramer, when compared to A2 \times FVB derived CD8 $^{+}$ T cells, and that A2 \times neu CD8 $^{+}$ T cells required 10–100 times more peptide to achieve comparable lysis than T cells from A2 \times FVB mice. The lower response of the CTL from A2 \times neu mice could not be attributed to low expression of TCR and CD8 molecules, indicating that the TCR on these CTL are of low affinity. Taken together, these results demonstrate that A2 \times neu mice are tolerant to neu antigens when compared to A2 \times FVB mice, and tolerance is achieved by the elimination of T cells with high avidity [25].

The low-avidity T cells that persist in A2 \times neu mice were able to recognize and kill tumor cells expressing HLA-2.1/neu antigens. We wanted to determine the antitumor potential of these cells. Previous studies have shown that plasmid DNA vaccination [26] or allogeneic cell vaccination [21] could induce a protective immune response in neu mice. In contrast to these studies, in which animals were immunized first and then challenged with the

tumor, we used a therapeutic tumor model whereby animals were first implanted with the tumor and then immunized. Our results demonstrate that, to generate a protective immunity in neu mice, the application of multiple immunizations is critical. In the adoptive transfer experiments, a single T cell transfer has a minimal effect on tumor growth; however, sequential adoptive transfers of T cells significantly delayed tumor growth. Similar results were found with immunization of DC pulsed with the p369–377 and p773–782 peptides. Single immunization inhibited 7% of tumor growth while three immunizations inhibited tumor growth by 25–30%. The application of multiple immunizations could have important implications for the design of immunotherapeutic strategies targeting self tumor antigens. The significance of applying multiple immunizations could be interpreted as follows: (1) it might be necessary to constantly restimulate the already activated immune response with professional APC, since the interaction of the low-avidity T cells and tumor cells might not be sufficient to actively maintain the effector function of tumor-specific T cells; (2) it might be possible that the stimulated tumor-specific T cells are further tolerized or deleted after initial interaction with the tumor, sequential immunizations could then activate new responses; or (3) as demonstrated by Cordaro et al. [27], the T cell response is capable of inducing an effective antitumor response only when a memory low-avidity T cell response is generated. It might be that the application of multiple immunizations in A2 \times neu mice generates a memory response, resulting in the most efficient strategy to induce tumor immunity in these mice.

Recently, Ercolini et al. [28] reported the identification of an H2D a -neu-restricted epitope. Two immunizations with

DC pulsed with this peptide prior to tumor challenge did not induce a protective immunity in *neu* mice. We have also observed a minimal protection (less than 10% of tumor growth inhibition) in animals that were immunized three times with DC pulsed with p369–377 or p773–782 peptides in the absence of IL-2 (data not shown). De Visser et al. [29] demonstrated that levels of IL-2 were markedly reduced in low-avidity T cells and that the effector function of these cells was severely impaired. Our data were in agreement with their results in that the induction of an antitumor response was dependent on the addition of IL-2. Interestingly, in contrast to anti-OX40 mAb, the addition of IL-2 did not significantly augment the number of tetramer-binding cells after DC immunization. The exact role of IL-2 in this model remains to be determined. The role of IL-2 could be to maintain the effector function of the T cell response [13, 14]. However, DC immunization in combination with anti-OX40 mAb has a synergistic effect, resulting in substantial tumor growth inhibition. Even though previous studies have demonstrated that injections of anti-OX40 mAb are sufficient to induce the eradication of established tumors [17, 18], the therapeutic effect of anti-OX40 mAb seems to be limited in the *neu* tumor model. This could be because of the low immunogenicity of the tumor in these mice. We do not yet know for certain how anti-OX40 mAb influences the immune responses. Anti-OX40 mAb might stimulate CD4⁺ T cells [15, 16] to provide help to CD8⁺ T cells and, as a consequence, stronger antitumor responses can be observed. The other mechanism in which anti-OX40 could exert its effect is by directly enhancing the CD8⁺ T cell responses, as demonstrated by De Smedt et al. [30]. The tetramer-binding and cytotoxic assays indicate that CD8⁺ T cell responses in A2×*neu* mice are enhanced in the presence of anti-OX40 mAb, and the increased presence of tetramer-binding cells correlates with the cytotoxic activity. These data suggest that the improvement of the immune response in the presence of anti-OX40 is most probably due to a greater number of tumor-specific T cells that were expanded after immunization with DC. Taken together, these data indicate that for the activation of an immune response in A2×*neu* mice, the use of costimulatory molecules such as IL-2 or anti-OX40 mAb might be critical to optimally stimulate/expand the low-avidity repertoire in order to generate antitumor immunity.

With the use of the A2×*neu* mice, we have established that tolerance in *neu* mice is a consequence of the deletion of high-avidity T cells. Although most of the effective treatments correlate with immune responses that are of high avidity, the observation that low-avidity T cells can recognize and kill tumor cells [9, 10, 27, 29] offers an opportunity to exploit these cells for the induction of antitumor responses. It is highly probable that immuno-

therapy alone under tolerant conditions will not be sufficient to induce the complete eradication of tumors. However, if the immune repertoire for a self tumor antigen is properly stimulated and used in combination with another form of therapy it could result in rejection of the tumor. In this regard, we have recently evaluated the combination of immunotherapy and antiangiogenic therapy. Our results demonstrate that the application of each therapy alone retarded tumor growth; however, when these therapies were combined they resulted in complete rejection of the tumor in *neu* mice [31]. These results further confirmed the antitumor potential of low-avidity T cells and the utility of this repertoire in antitumor responses. In these studies, we demonstrated that if the immune responses from tolerant hosts were properly primed and the effector function was maintained over time, the immune response would contribute to the destruction of the tumor. Further evaluations of the low-avidity repertoire against self tumor antigens will ultimately lead to improvements of the strategies for the optimization of vaccination immunotherapies against self tumor antigens.

4 Materials and methods

4.1 Mice and cell lines

The *neu* transgenic mice (line 202) were commercially obtained from Jackson Laboratories (Bar Harbor, ME) and maintained homozygously. The FVB mice were purchased from Harlan (Indianapolis, IN). The HLA-A2.1/Kb transgenic mice were kindly provided by Dr. Linda Sherman from the Scripps Research Institute (La Jolla, CA). The *neu* and FVB mice were mated with the HLA-A2.1/Kb mice to generate A2×*neu* and A2×FVB mice. The N202.A2 cell line was established from a spontaneous tumor. The tumor was digested with a mixture of 1 mg/ml collagenase, 10 U/ml hyaluronidase and 20 µg/ml DNase (all from Sigma, St. Louis, MO). Cells were grown in RPMI 1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 5×10⁻⁵ M 2-mercaptoethanol (2-ME) and 50 µg/ml gentamicin. Cultured cells were treated by differential trypsinization to remove fibroblasts. The N202 mammary cell line was obtained from Dr. Pier-Luigi Lollini (University of Bologna, Bologna, Italy). The T2-A2.1/Kb cell line was provided by Dr. Linda Sherman. Anti-OX40 (OX86) mAb were obtained from the European Cell Culture Collection (Wiltshire, GB). All cell lines were maintained in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 5×10⁻⁵ M 2-ME and 50 µg/ml gentamicin.

4.2 Flow cytometry analysis

The N202.A2 and N202 cells were stained with primary antibodies (0.5 µg/ml) against the human HLA-A2 antigen

(PA2.1), murine H2D^b/H-2L^a (KH117; PharMingen, San Diego, CA) and rat neu (neu-Ab-4; Calbiochem, San Diego, CA). To measure the expression of CD8 and TCR β molecules on CTL, the anti-CD8 α -FITC (53-6.7; PharMingen) and anti-TCR β -FITC (H57-597; PharMingen) were used, respectively. Samples were analyzed in a FACScan (Becton Dickinson, San Diego, CA) and data analysis was performed using CellQuest software.

4.3 Generation of DC and immunization of A2xneu and A2xFVB mice

For DC isolation, magnetic beads conjugated with anti-CD4, anti-CD8 and anti-B220 (Dynal, Oslo, Norway) were used to deplete bone marrow cells of lymphocytes. The remaining cells were cultured in complete RPMI medium containing 3% GM-CSF (supernatant from J558L cells transfected with the murine GM-CSF gene obtained from Dr. R Steinman, Rockefeller University, New York, NY). For DC maturation, 100 U/ml TNF- α was added on day 7 and DC were collected on day 9. Maturation of pulsed DC was confirmed by evaluating the expression of B7-1, B7-2, MHC class I and MHC class II. Mature DC were pulsed with the p369-377 or p773-782 peptides at 10 μ g/ml for 3 h at 37°C.

4.4 Stimulation of p369-377- and p773-782-A2.1-neu-restricted CTL responses

Ten days after immunization with DC, animals were killed and spleens were removed. For stimulation of cultures, T cells (10 6 cells/well) were incubated with autologous irradiated (3,000 rad) LPS spleen blasts (2 \times 10 5 cells/well) that were pulsed with the p369-377 (KIFGSLAFL) and p773-782 (VMAGVGSPYV) peptides in 24-well plates. After 5 days, CTL were assayed for lytic activity. The N202.A2, N202 and T2-A2.1/Kb cells pulsed with the peptides were incubated with 150 μ Ci sodium [51 Cr]chromate for 1 h at 37°C. Cells were washed three times and resuspended in complete RPMI medium. For the cytotoxic assay, [51 Cr]-labeled target cells (10 4) were incubated with varying concentrations of effector cells in a final volume of 200 μ l in U-bottom 96-well microtiter plates. Supernatants were recovered after 6 h of incubation.

4.5 Tetramer staining

The A2.1-p369-377-PE and A2.1-p773-782-PE tetramers were obtained from the NIAID Tetramer Core Facility. Spleen cells from mice immunized with the p369-377 and p773-782 peptides were stained with the A2.1-p369-377-PE and A2.1-p773-782-PE tetramers (2 μ g/sample) for 1 h at room temperature and then with anti-CD8-FITC for an additional 30 min at 4°C. Samples were analyzed in a FACScan.

4.6 Adoptive Transfer

The CTL lines specific for p369-377 and p773-782 peptides derived from A2xneu or A2xFVB mice were expanded *in vitro* by mixing 2 \times 10 6 cultured T cells with 2 \times 10 6 irradiated autologous LPS spleen blast cells pulsed with the p369-377 or p773-782 peptides containing 25 U/ml IL-2 in T25 flasks in a total volume of 10 ml. CTL were tested for cytolytic activity after *in vitro* stimulation. To test the effect of adoptively transferred T cells, A2xneu mice were implanted s.c. with 10 6 N202.A2 tumor cells. On day 7 after tumor inoculation, animals were randomly divided into groups of five, and 10 7 stimulated CTL were directly injected into the tumor. Animals received daily i.p. injections of IL-2 for 1 week (10 4 IU/day). Tumor growth was monitored every 5 days and growth rates were determined by caliper measurements in two diameters. Tumor volume was expressed as: (minor diameter) 2 \times major diameter/2. Statistical analysis was determined by Student's *t*-test.

4.7 Immunization

A2xneu mice were implanted s.c. with 10 6 N202.A2 cells on day 0. On day 7, animals were randomly divided into groups of five and immunized once, twice or three times s.c. with 10 6 DC pulsed with the p369-377 or p773-782 peptides in the presence or absence of rIL-2. Animals that received rIL-2 were injected with 10 4 IU/day for different periods of time as described in the experiments. Anti-OX40 (100 μ g/injection) was injected 2 days after DC immunization and once a week thereafter.

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